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Bcl-2 overexpression prevents apoptosis-induced Madin-Darby canine kidney simple epithelial cyst formation[1]

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Bcl-2 overexpression prevents apoptosis-induced Madin-Darby canine kidney simple epithelial cyst formation.

Background. Madin-Darby canine kidney (MDCK) cells develop into simple epithelial cell cysts when cultured in type I collagen gel. We found that MDCK cells initially grow into multilayer cell aggregates and subsequently develop central lumen that contain apoptotic cells. We hypothesized that apoptosis might be essential for the formation of MDCK cysts.

Methods. Using MDCK cells cultured in collagen gel as the experimental model, we investigated how renal cells organize to form cysts. To delineate the role of apoptosis in the process of cyst formation, MDCK cells were transfected with the *bcl-2* gene. Characterization of apoptosis was studied by morphological and biochemical methods.

Results. Bcl-2 overexpression conferred resistance to apoptosis. Cultured in collagen gel, Bcl-2 transfectants rarely formed a simple epithelial cyst, but instead remained as a multilayer cell aggregate containing central or multiple lumens, or even developing into branching structures.

Conclusions. Because Bcl-2 overexpression averts cyst cavitation, these data clearly indicate that apoptosis is an essential initial event for renal cyst formation.

It is our current understanding that apoptosis, so-called programmed cell death, plays important roles in the homeostatic control of cell number to maintain tissue size and functions [1]. During the developmental process of the kidney, apoptosis can be observed during normal differentiation of the metanephros [2, 3]. In addition, apoptosis has also been found in either regenerating or regressing renal tubule cells during and after ischemia-induced acute tubular necrosis [4, 5]. The genes that confer the execution of apoptosis have gradually been unveiled. Among them, *ced-3* and interleukin-1β-converting enzyme are genes that confer the execution of apoptosis [6–8]. On the other hand, *ced-9* and B-cell lymphoma-2 (*bcl-2*) are genes that confer resistance to apoptosis [9–11].

[1] **See Editorial, p. 334.**

Key words: renal cystic disease, apoptotic cells, programmed cell death, metanephros, polycystic kidney disease, ischemia.

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The renal cyst is a pathological structure in which a single layer of renal epithelial cells forms a wall around a fluid-filled cavity. Experimental studies have demonstrated that tubular cell hyperplasia, fluid accumulation, and abnormalities of the basement membrane play important roles in renal cyst formation [12]. However, an increasing amount of evidence has linked cell death to renal cystic diseases. A recent report indicated that widespread apoptosis was found in the tubulointerstitial areas of kidneys of autosomal dominant or recessive polycystic kidney disease [13]. It is therefore suggested that the apoptosis of tubular epithelial cells is related to the progressive failure of renal functions in polycystic kidney disease (PKD). In addition, three studies using transgenic mice have shown that knocking out the *bcl-2* gene led to the development of cystic kidney [14–16]. These studies indicate that apoptosis is involved in the pathogenesis of renal cystic diseases. However, whether apoptosis is involved in the development of renal cysts remains to be determined. With the understanding that the task of using human specimens or animals with cystic kidneys to study the original formation of a renal cyst is quite difficult, we employed *in vitro* studies. To better understand the underlying cellular events during the initial phase of cyst formation, we conducted a series of studies with Madin-Darby canine kidney (MDCK) cells cultured in collagen gel, which has been established as a model for studying cell organization of spherical cyst formation [17–20].

We observed that MDCK cells initially grew into solid cell aggregates and subsequently developed a central lumen that contained apoptotic cells. Interestingly, apoptosis occurred before and during the process of cyst development. To delineate the causal relationship between apoptosis and cyst formation, we transfected MDCK cells with the *bcl-2* gene. The data showed that Bcl-2 overexpression almost completely averted the formation of simple epithelial cysts in collagen gel. Our results indicate that apoptosis plays an important role in cyst cavitation at the initial step of cyst formation.

METHODS

Cell line

Madin-Darby canine kidney cells (ATCC, Rockville, MD, USA) were regularly maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in humidified 5% CO_2 atmosphere.

Preparation of hydrated collagen gel and cultures in three-dimensional gel

Type I collagen was prepared from rat tail tendons by modification of an established procedure [21]. For the preparation of collagen gel, three volumes of collagen stock were mixed with 10× medium F12 (1 vol), 5% $NaHCO_3$ (0.5 vol), 0.1 m HEPES (1 vol), 0.2 m $CaCl_2$ (0.1 vol), 1n NaOH (0.1 vol), and 4.3 volumes of dispersed cell suspension in culture medium. Mixtures of cells (2×10^5) and collagen gels (1 ml) were plated in 35-mm dishes, and the cultures were placed in an incubator (5% CO_2 in air, 37°C) to allow for the gelation. Each culture was then overlaid with 1.5 ml of culture medium, which was then replaced every other day.

Histological studies

Cells cultured in type I collagen gel were fixed with formalin, embedded in paraffin, sectioned, deparaffinated, and stained with hematoxylin-eosin according to standard histological methods. For fluorescence examination of nuclear morphology to evaluate apoptosis, the deparaffinated sections were extensively rinsed in phosphate-buffered saline solution (PBS), were then stained with Hoechst 33258 (5 μg/ml) for 60 minutes in the dark, and were visualized under a fluorescence microscope (Nikon, Tokyo, Japan).

Electron microscopic examination of the MDCK cyst

Madin-Darby canine kidney cysts developed in collagen gel were harvested by treating the gel with 1 mg/ml collagenase. After digestion, MDCK cysts were collected by centrifugation and were then fixed with glutaraldehyde (2.5% wt/vol in 0.1 M Sorenson's phosphate buffer, pH 7.4) for two hours at room temperature. The cyst pellets were washed with the buffer and postfixed in 1% osmium tetraoxide for one hour. Samples were dehydrated and embedded in epon resin. Sections (100 nm) were mounted on carbon/formar-coated grids and were stained with uranyl acetate for 10 minutes, followed by saturated lead citrate for four minutes before being examined under a transmission electron microscope.

Extraction of low molecular weight DNA and electrophoresis

The method for extraction of low molecular genomic DNA has been described previously [22]. Briefly, low molecular genomic DNA was extracted from cultured cells with 0.5% Triton X-100, 10 mM ethylenediaminetetraacetate, and 10 mM Tris, pH 7.4, and phenol/chloroform. The DNA was precipitated in propanol and was electrophoresed by a 1.5% agarose gel. Finally, the DNA was visualized with ethidium bromide staining under ultraviolet light.

Cell-cycle analysis

The apoptosis was quantitated by flowcytometry using propidium iodide as Nicoletti et al described [23]. MDCK cells were first treated with dispase (0.6 U, 0.5 μg/ml PBS) to obtain a better cell suspension. The dispase-digested cells were then washed with PBS and were fixed in 70% alcohol. After fixation, cells were treated with RNAse (100 μg/ml PBS) and stained with propidium iodide (40 μg/ml PBS). The mixed cells were incubated in the dark at room temperature for 30 minutes and were analyzed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA, USA) with an excitation set at 488 nm. Data were analyzed by Cell FIT software and were represented as either histograghs or numbers. The hypodiploid DNA peak of apoptotic cells can be distinguished from the normal diploid DNA peak on the fluorescence profiles of propidium iodide-stained cell.

Transfection

The DNA construct used for the transfection was a gift from Dr. Tsujimoto [10]. The vector pCΔj contained the G418 resistance gene and the EBV-derived replication origin. The *bcl-2* cDNA sequences were expressed by simian virus 40 enhancer/promoter regulatory elements (pCΔj-*bcl-2*). A DNA construct without *bcl-2* cDNA sequences (pCΔj-SV2) was used as a control. Transfection of MDCK cells was done with the method of lipofection. In brief, DNA-liposome complexes were applied to MDCK cells cultured to approximately an 80% to 90% confluence for 24 hours in a CO_2 (5%) incubator. The ratio of DNA/liposome complex at 1 μg/20 μl lipofectamine was used to obtain the optimal result for transfection. Following the transfection, culture medium was replaced with 10% FCS/DMEM. At 72 hours after transfection, cells were passaged by 1:10 dilution into G418 selective medium (effective concentration, 0.5 mg/ml).

Western blot analysis

Expression of Bcl-2 in wild-type MDCK and Bcl-2 transfectants was determined by immunoblotting, as described previously [24]. In brief, 100 μg cell homogenate protein from specific samples was resolved by 10% SDS-PAGE and was electrophoretically blotted onto nitrocellulose paper. The nitrocellulose paper was incubated with mouse antihuman Bcl-2 polyclonal antibody (Dako, Carpinteria, CA, USA), and immunocomplexes were

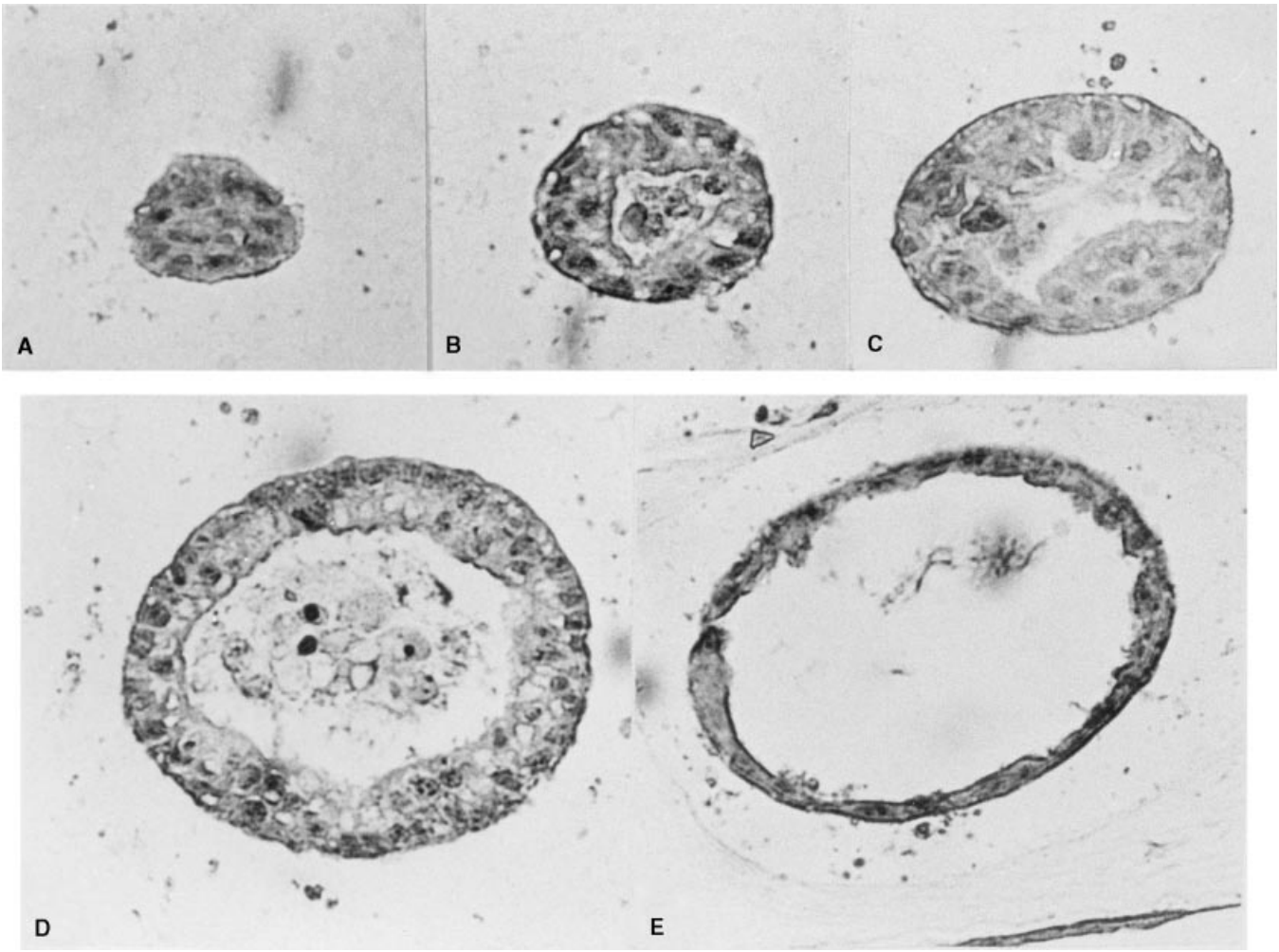


Fig. 1. Histologic examination of the developmental process of MDCK cyst formation. (*A*) Day 3 early cell aggregate. (*B*) Initiation of cyst formation associated with cell degeneration within the cystic cavity at day 4. (*C*) Early cyst in which the cavity has just been developed at day 5. (*D*) Overt cyst containing central apoptosis at day 7. (*E*) Simple cuboid epithelial cell cyst at day 8 (bar indicates 50 μm).

detected with horseradish peroxidase-conjugated goat antimouse IgG antibody (1:1000 dilution); finally, the immunocomplexes were made visible by fluorography with an enhanced chemiluminescence detection kit (Amersham International PLC, Buckingham, UK).

Assessment of urokinase-type plasminogen activator activity

To assess urokinase-type plasminogen (uPA) activity, cells were cultured to confluency and were then serum starved for 24 hours. Both cell lysate and conditioned medium were harvested and stored in a freezer prior to the assay. For the assay, 0.1 μm plasminogen and 0.3 μm S-2251 (leu-lys-val-PNB) were added to the samples in ELISA plates. Activated uPA catalyzes plasminogen into plasmin, which further cleaves S-2251 into a spectrometer-detectable product. The reaction mixtures were read by the ELISA reader at 405 nm absorbance with a 15-minute interval, and the results were analyzed.

Assessment of cell proliferation

Estimation of proliferation rate of MDCK cells and Bcl-2 transfectants was assessed by measuring the (*a*) cell number and (*b*) thymidine incorporation in cultures. Cells were plated at the density of 1×10^5/dish on 60-mm dishes for one day, and then media were changed every two days. Cells were harvested by trypsinization, and the cell number was counted by hemocytometer. To assess thymidine incorporation rate, 2 ml of [^{3}H]thymidine (1μCi/ml) were added to each of the dishes and they were incubated at 37°C for four hours. The incorporation was terminated by dilution with 1 ml of cold thymidine (100 μg/ml). DNA was precipitated with cold 30% trichloracetic acid and washed once with 5% trichloracetic

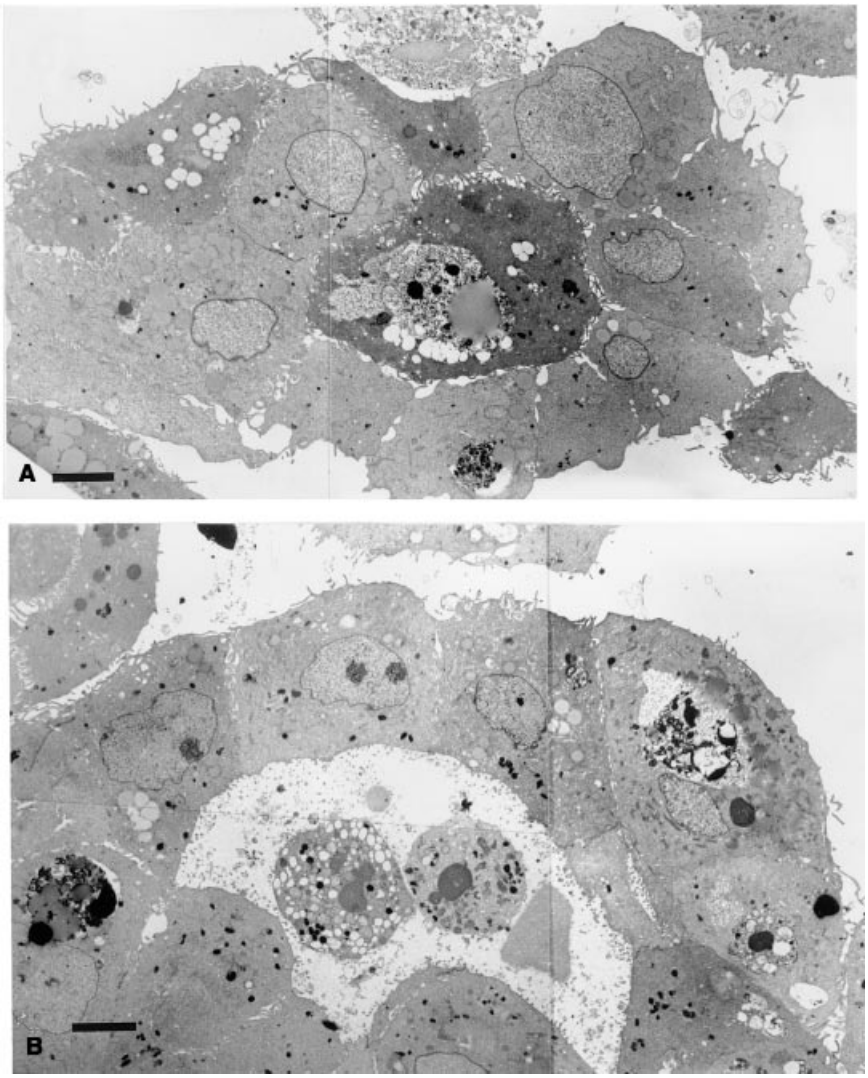


Fig. 2. Transmission electron microscopic examination of MDCK cell aggregate (*A*) and cysts (*B*) in which the cavity has just been developed. (A) A solid cell aggregate with central cell apoptosis. The cell in the center of the cell mass exhibits nuclear condensation and fragmentation. (B) In the newly formed cyst cavity, there are two cells showing nuclear condensation and fragmentation. In addition, engulfment of apoptotic bodies is observed in the surrounding epithelial cells. The polarity of lining epithelial cells of the cyst exhibits microvilli facing to the cyst lumen and also unfolded basolateral membrane resting on the collagen gel (bar indicates 4 μm).

acid, then 95% ethanol. [^{3}H]DNA was measured using a Beckman Model LS 6800 scintillation counter.

RESULTS

Developmental process of MDCK cysts in collagen gel

When MDCK cells were cultured in collagen gel, they underwent clonal growth within a few days [17–20]. At days 3 to 5, MDCK cysts developed and then gradually expanded, the diameter of which reaching 200 μm within two weeks.

To study how a cyst is formed, we first examined the morphology of MDCK cells from day 1 to day 8 in detail. We found that MDCK cells formed a solid aggregate or cell mass within the first three days in collagen gel and developed a cyst cavity later (Fig. 1 A–D). Pictures of cell degeneration, as manifested by nuclear condensation and degradation of cellular contents inside the cell mass were the first clues of morphological changes observed (Fig. 1 B, C). Although the cavity became larger as the cyst grew, clear pictures of cells undergoing apoptosis were still frequently observed inside of the cyst (Fig. 1D). Further examinations by transmission electron microscope showed consistent results (Fig. 2). We observed both obvious pictures of apoptosis in the cyst cavity and engulfment of apoptotic bodies by the surrounding epithelial cells (Fig. 2). In addition, approximately when the cyst cavity was formed, the epithelial cells lining the cyst exhibited a polarity with the microvilli facing the cyst lumen and unfolded basolateral membrane in the collagen gel (Fig. 2B). Such a polarity is similar to that reported previously by Wang, Ojakian and Nelson, and is considered to be characteristic for the epithelial cells lining a lumen [20]. Among all of the MDCK cysts examined during this stage, apoptosis was seen in their cyst cavity without exception.

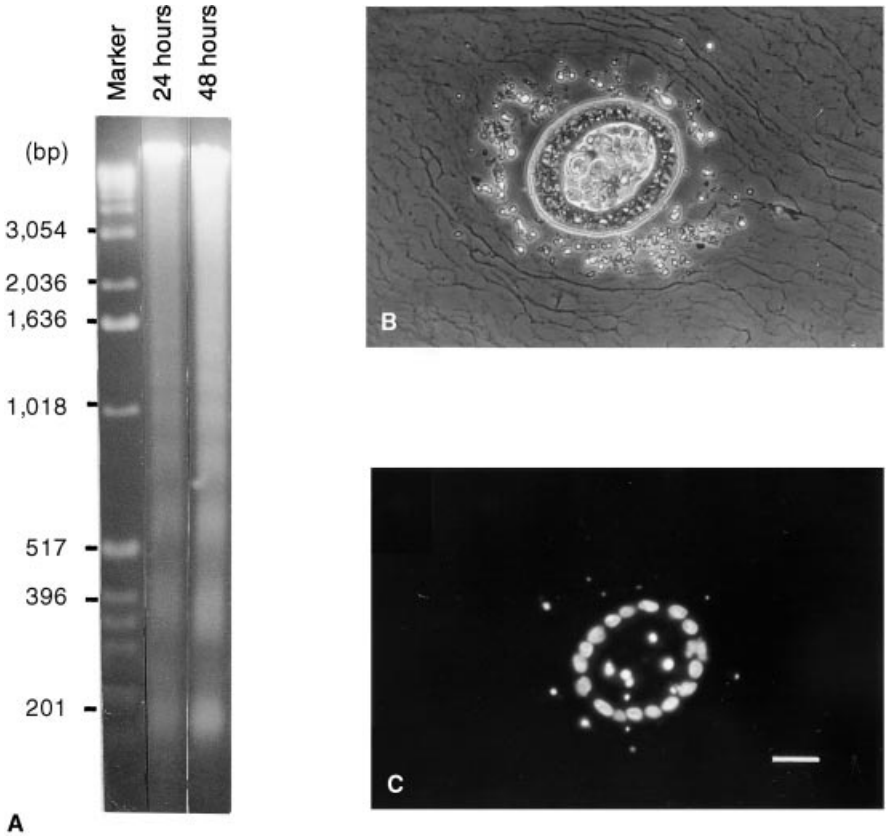


Fig. 3. Demonstration of the presence of apoptosis during cyst formation. (*A*) Results of electrophoretic study of DNA extracted from MDCK cells cultured in collagen gel for 24 and 48 hours. (*B*) Phase contrast and (*C*) fluorescence micrographs of MDCK cysts cultured in collagen gel for 4 days. The whole gel was fixed, embedded in the wax and sliced by the microtone at the thickness of 5 mm. Finally, MDCK cysts were stained with Hoechst 33258 and examined under fluorescence microscopy (bar indicates 50 μm). Apparently there were condensed and fragmented nuclei inside the MDCK cyst cavity.

The cell death inside the cyst cavity was further confirmed to be apoptosis by biochemical methods and fluorescence microscopical examination. Biochemical study demonstrated that the DNA ladder appeared as early as two days in collagen gel culture (Fig. 3A). Figure 3 B and C are pictures of the same MDCK cyst examined under phase-contrast and fluorescence microscope, respectively, after Hoechst 33258 staining. Condensed and fragmented nuclei were observed inside the cyst. This result is consistent with the histological and electron microscopical findings. Taken together, these data show that apoptosis was involved in the development of the MDCK cyst cavity.

Bcl-2 transfectants

It has been shown that Bcl-2 overexpression prevented cell death from various types of induction [1]. To explore the causal relationship between apoptosis and the cyst cavitation, we next transfected MDCK cells with *bcl-2*. Several stable transfectants (B1, B4, B6, and B9) were obtained, which all exhibited markedly induced levels of Bcl-2 protein (Fig. 4), as determined by Western blot analysis.

To assess the growth characteristics of the wild-type MDCK cells and the Bcl-2 transfectants, cells were cultured at the density of 1×10^5 per 60-mm dish with

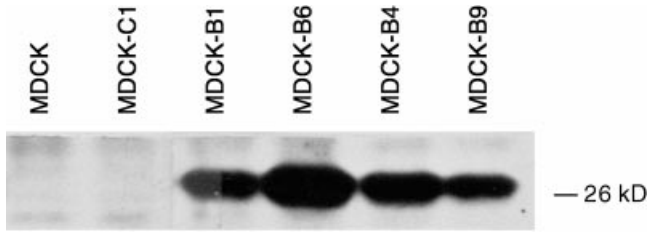
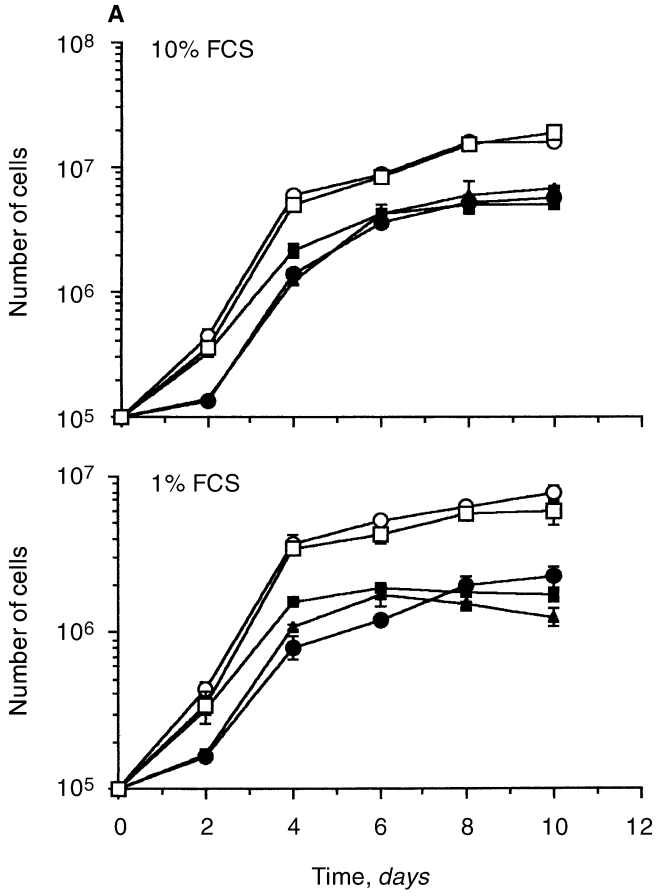


Fig. 4. Bcl-2 expression in MDCK cells transfected with the *bcl-2* gene. Western blot analysis was employed to demonstrate the expression of Bcl-2 in homogenates of wild type, plasmid control (C1) and *bcl-2* transfected (B1, B4, B6, and B9) MDCK clones.

DMEM containing 10% or 1% FCS. Their cell numbers were assessed within 10 days of culture. We found that Bcl-2 transfectants exhibited a higher density of cells during the confluent phase than wild-type MDCK cells or control transfectants, regardless of the percentage of FCS (Fig. 5A).

At the confluent phase, wild-type MDCK cells formed domes, a differentiated function for transporting renal epithelial cells, and gradually shed cells into the media from the monolayer (data not shown). Assessment of the floated cells by FACscan revealed that more than 90% of them are apoptotic, indicating that anoikis could be the cause [25, 26]. In contrast, Bcl-2 transfectants



developed very compact monolayers that exhibited augmented cell clumping and even tubing with a lesser degree of cell floating (Fig. 5B). Because thymidine incorporation rates in these cultures did not vary, the higher cell density manifested in Bcl-2 transfectants at the confluence was due to an increase in the compactness of the culture rather than enhanced cell proliferation. Previously, we described that overconfluence triggered cell shedding and apoptosis, and coined the action "confluent cell death" [26]. Here, we determined whether Bcl-2 conferred resistance to confluent cell death. As expected, the control transfectant developed floating and apoptosis that was manifested by the presence of DNA ladder in the agarose gel electrophoresis. In contrast, Bcl-2 transfectants remained adherent to the culture plates at this phase and were resistant to apoptosis. Thus, no nuclear or DNA fragmentation could be observed (Fig. 5C). These results indicate that Bcl-2 transfectants are resistant to the apoptosis resulting from either a lower serum concentration or overconfluence.

Morphogenesis of Bcl-2 transfectants in collagen gel

Finally, Bcl-2 and control transfectants were cultured in collagen gel. The control plasmid transfectants behaved very much like the wild-type MDCK cells, exhibiting DNA fragmentation (data not shown). Morphologically, the control transfectants also developed cysts when cultured in collagen gel within five to eight days (Fig. 6 A, D). In contrast, the majority of Bcl-2 transfectants developed into cell aggregates and not definitive cysts

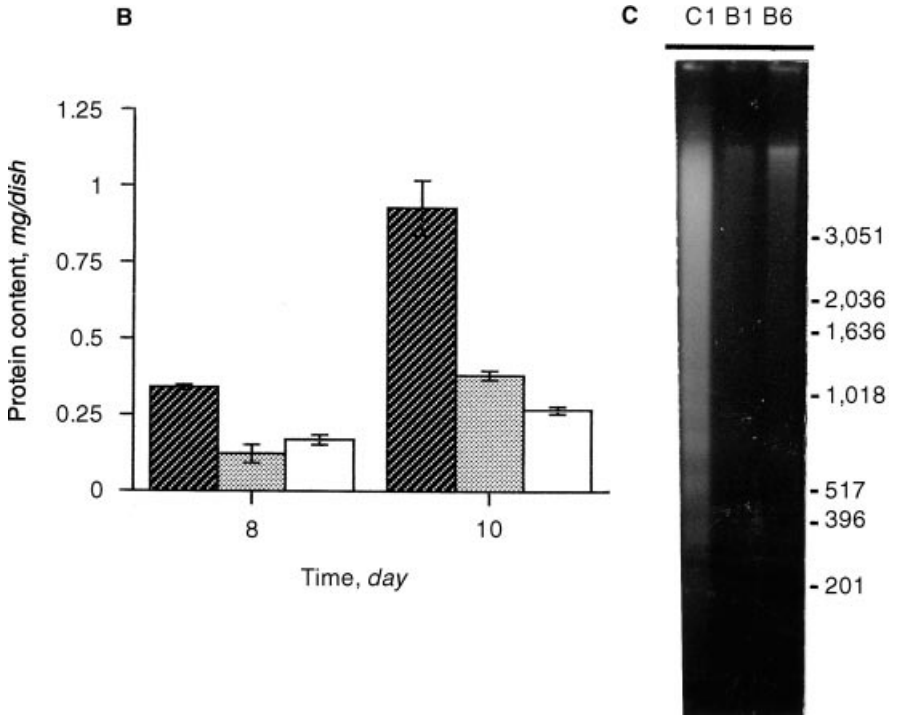


Fig. 5. Growth analysis of different MDCK clones. (*A*) Cells plated at 1×10^5 per 6-cm dish were cultured in growth media containing 10% and 1% FCS. Symbols are: (■) MDCK; (▲) C1; (●) C2; (○) B1; (□) B6. The number of cells represents the mean of two experiments in triplicate. (*B*) The total protein contents of floated cells collected at day 8 and 10. Symbols are: (▨) C; (■) B1; (□) B6. Despite the crowded appearance during confluency, levels of floated cells are reduced in B clones. (*C*) Electrophoresis of DNA extracted from floated cells collected at day 10. Only C1 cells exhibited a DNA ladder.

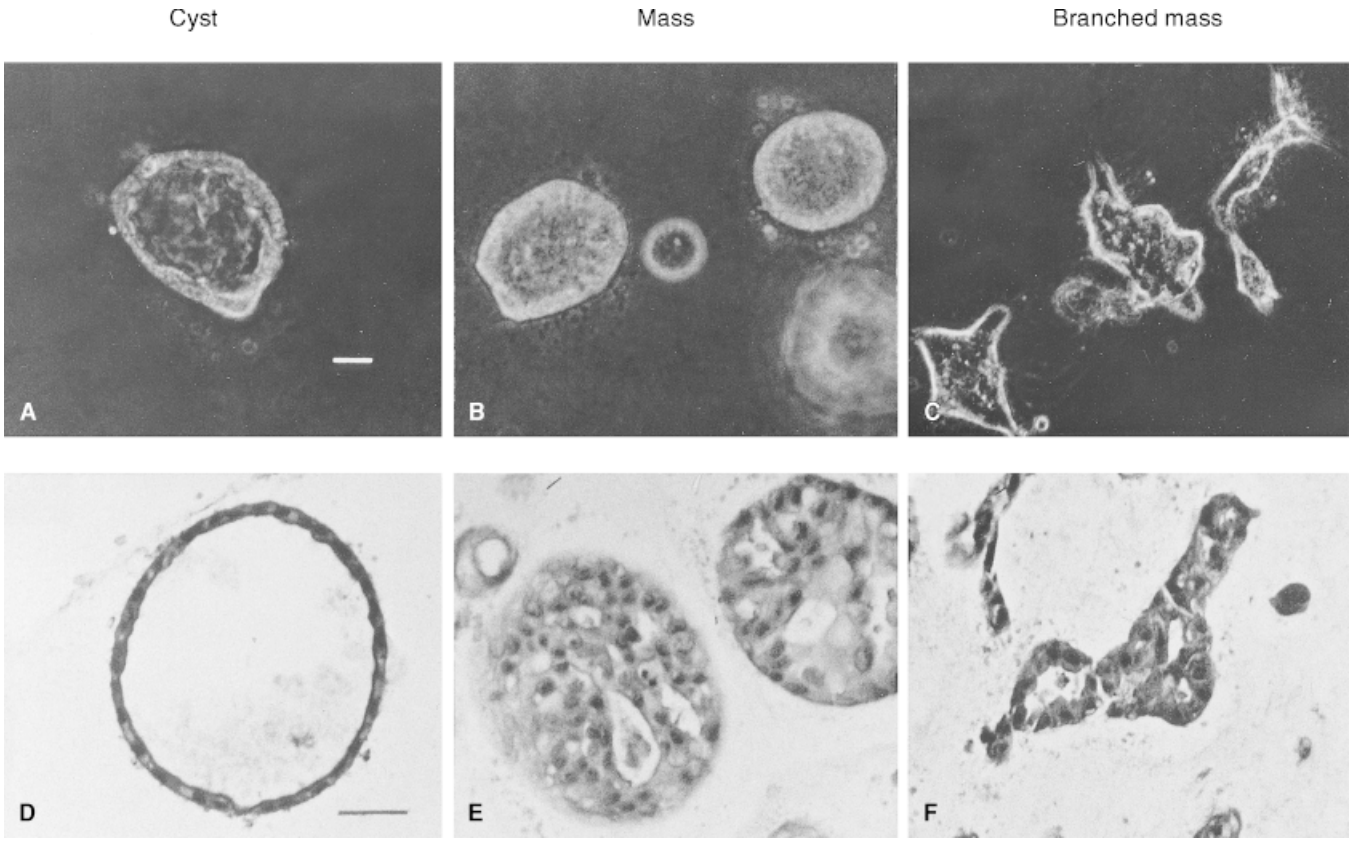


Fig. 6. Morphology of control and Bcl-2 transfectants cultured in collagen gel for 8 days. Upper panel shows phase-contrast micrographs taken under light microscope with magnification of ×200. Lower panel shows hematoxylin and eosin stained pictures of cyst, cell mass, and branched tubule-like structures (bar indicates 50 μm). Notice that central or multiple lumen are formed in the cell aggregate.

at the same stage (Fig. 6 B, E). Under phase-contrast light microscopic examination, it was calculated that greater than 85% of cell colonies of B6 and B9 and 70% of B1 and B9 formed round cell aggregate in collagen gel at day 8 (Fig. 7). Unexpectedly, approximately 22%, 9%, and 8% of B1, B4, and B9 cell colonies, respectively, formed a branched mass that manifested tubule-like structures, as shown in Figure 6C. As examined histologically, these branched tubule structures contained a central lumen (Fig. 6F) and exhibited similar morphology of a renal tubule under cross section (data not shown). To further examine the morphology of Bcl-2 transfectants cultured in collagen gel, we employed electron microscopical studies. At the initial stage (days 3 to 5) in collagen gel, neither apoptosis nor obvious lumen could be found in the small cell aggregate. However, clues that cell aggregates were developing lumen with microvilli facing to it could be observed at day 5, as shown in Figure 8. As these cell aggregates grew larger, they developed multiple overt lumen with a few apoptototic cells inside (Fig. 6E). Obviously, Bcl-2 overexpression does not change the capacity of MDCK-cell remodeling to form lumen in response to collagen gel.

Activation of uPA has been implicated in the mechanisms of MDCK branching tubulogenesis [27]. We examined the uPA activity from both cell lysate and condition medium of various types of cells cultured to confluence and cells that were serum starved for one day. The uPA activity of cell lysates was not different in all lines examined, ranging from about 2.0 to 2.5 pmole/mg protein. However, we found that uPA secretion varied in the different lines (Fig. 9). The levels of uPA release in Bcl-2 transfectants were in the following order: B1 > B4 = B9 > B6, indicating that these transfectants were from heterogeneous populations. Because the levels of secreted uPA, but not Bcl-2, parallel to the degree of branching tubule morphology, these results rule out the possibility that Bcl-2 contributes directly to the branching tubule morphology. In addition, control transfectants showed less cyst formation than wild-type MDCK, and more cell mass and a small percentage of branched structure (Fig. 7). Because C1 cells release a higher amount of uPA than wild-type MDCK, this phenotype may enhance the digestion of the adjacent collagen matrix and thereby contribute to the increased ratio of cell mass or even branching structure.

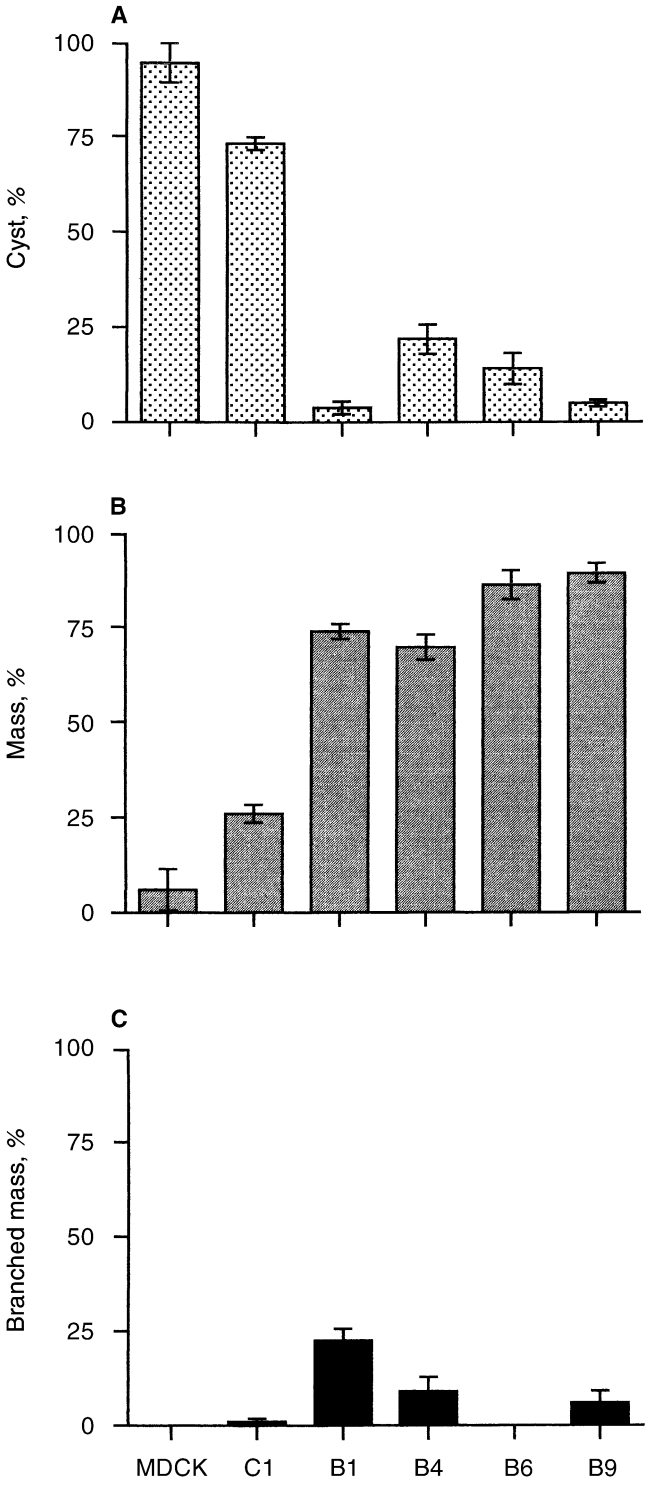


Fig. 7. Quantitative assessment of the morphology of MDCK clones C1, B1, B4, B6 and B9 cultured in collagen gel for 8 days. The majority of B clones formed cell aggregates in collagen gel. However, about 22%, 9%, and 6% of the B1, B4 and B9 cells, respectively, developed branched mass/tubule structures. Each bar indicates mean ± SE of 4 experiments in duplicate.

DISCUSSION

It is well established that MDCK cells could undergo membrane remodeling to form lumenization in response to collagen gel overlay [28, 29]. The lumenization is a structural result of cell remodeling, which requires at least uniform polarization of and tight junction formation between cells. In collagen gel, MDCK cells can also develop a closed lumen, which tends to be enlarged by both proliferation and fluid secretion of the lining epithelium or perhaps other mechanisms. In this study we demonstrate for the first time, to our knowledge, that apoptosis of renal epithelial cells plays important roles in the morphogenesis of renal cysts. First, apoptosis started earlier than the formation of the cyst cavity. Second, evidence of apoptosis was observable inside the cavity of all of the early cysts examined. Third, during cyst expansion, apoptotic bodies could still be found either in the cavity or on the lining epithelium of the cyst. Finally, the strongest evidence for the causal relationship of apoptosis and cyst formation is that when apoptosis is prevented, MDCK cyst formation could be averted. Taken together, apoptosis is therefore essential for the development of MDCK cyst cavitation. The morphogenesis of renal cyst cavity is particularly similar to recent reported mechanisms for the development of embryonic cavitation [30].

The cause of MDCK cell apoptosis in collagen gel remains to be determined. However, it has been shown that epithelial cells deprived of interaction with the underlying matrix would develop apoptosis within six to eight hours, a phenomenon recently described as anoikis [25, 31]. In view of the three-dimensional structure of the cell aggregate in gel, we speculate that epithelial cells in direct contact with extracellular matrix tend to survive, whereas those in the center may undergo apoptosis because of the deprivation of the cell–matrix interaction. On the other hand, we have characterized that MDCK cells cultured under type I collagen gel gradually developed apoptosis, and have defined this phenomenon as disoriented cell death [32]. It is possible that apoptosis can be initiated by limited space for cell anchorage, a phenomenon similar to anoikis, or result from a mismatch between confluent epithelial cells and type I collagen. In addition, recent reports indicated that the limitation of cell migration triggered apoptosis of endothelial cells despite the presence of extracellular matrix [33]. It is also possible that cells in the center of cell aggregates develop apoptosis because of a restriction of cell migration.

The examination of Bcl-2 overexpressed cells cultured in collagen gel revealed that they might account for two types of morphology during renal development. The epithelial cell aggregate with clues of lumen formation resembles the metanephric condensates during renal onto-

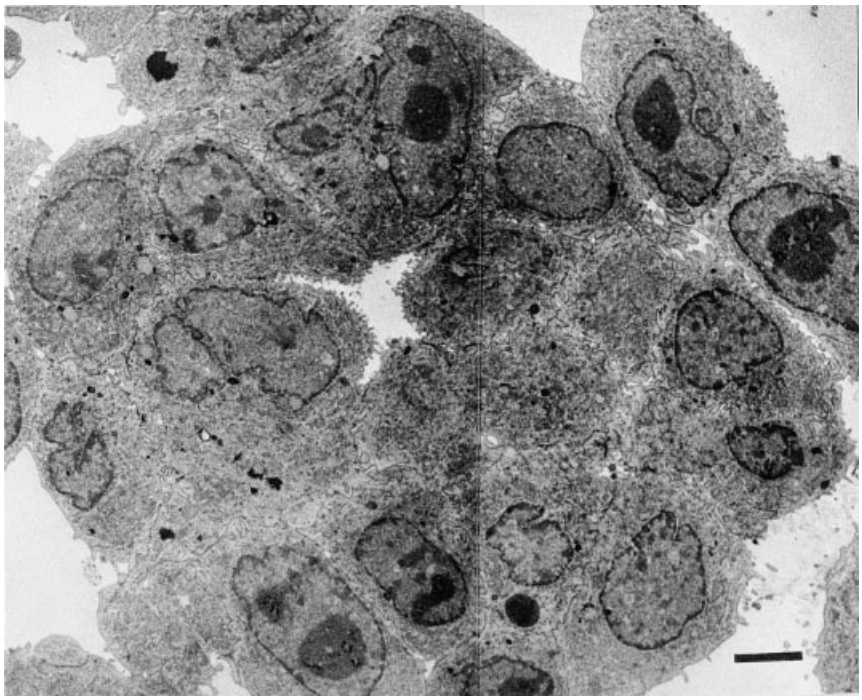


Fig. 8. Transmission electron microscopic examination of B1 cell aggregate formed in collagen gel for 5 days. A small but obvious lumen is formed in the cell mass (bar indicates 4 μm).

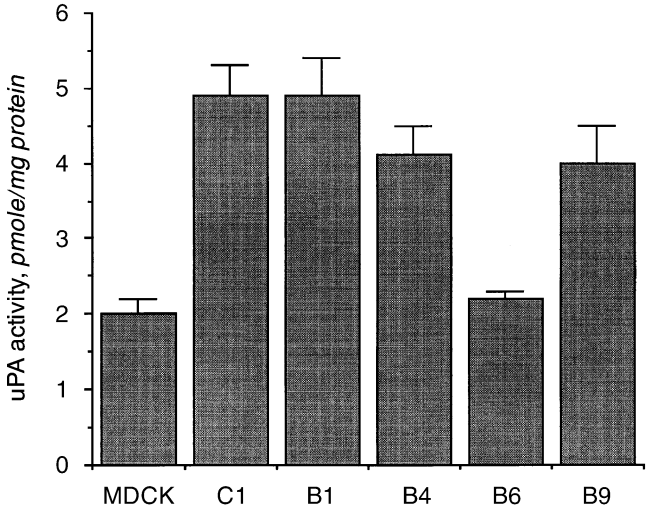


Fig. 9. Activity of secreted uPA in conditioned medium of different Bcl-2 transfectants. Each bar indicates mean ± SE of 4 experiments in duplicate.

genesis [34], where Bcl-2 levels are initially elevated at this stage [35, 36]. On the other hand, the branched tubule/mass represents for terminally differentiated distal nephron. Certainly, the characterization of these cells in more detail is required for the determination of their phenotype. Although the cause of the marked elevation of Bcl-2 expression in metanephric condensate during development is still not understood, our observations strongly indicate the functional role for Bcl-2 expression in the development of renal epithelial condensate and formation of renal tubule. Furthermore, this study also explains explicitly why homologous deficiency of Bcl-2 led to the development of cystic kidney [14–16]. One can argue that the development of MDCK cysts may not be similar to that of renal cysts in polycystic kidney disease. However, an increasing amount of evidence has also linked apoptosis to this disease. For example, a recent report indicated that in the kidneys of autosomal dominant PKD (ADPKD) and autosomal recessive PKD (ARPKD), widespread apoptosis was found [13]. Our laboratory also found that massive apoptosis could be detected in the cavity of renal cysts in ARPKD kidneys (unpublished data). Taken together, appropriately regulated apoptosis appears to be necessary for the morphogenesis of normal kidney structure.

Previously, Montesano, Schaller and Orci discovered that soluble factors produced by cultured fibroblasts could induce branching tubule morphogenesis of MDCK cells in collagen gel [19]. They consequently identified that hepatocyte growth factor (HGF) was the fibroblast-derived molecule that induced branching tubule by MDCK cells [37]. In contrast, other types of peptide growth factors did not confer this morphogenic effect. It is of interest that Bcl-2 overexpression and HGF both facilitate the formation of renal tubule by MDCK cells, but the morphology of these tubules is somewhat different. HGF induces branching tubule morphogenesis, which accounts for the cortical collecting tubule as proposed by Cantley et al [38], whereas Bcl-2 overexpression

facilitates the development of elongated tubule or mass with less branching. To probe the cellular mechanisms of HGF, Pepper et al observed that an increase in both urokinase activity and expression of urokinase receptor mRNA induced by HGF was involved in the effect of branching morphogenesis [39]. On the other hand, a recent report by Lu et al demonstrated that Bcl-2 overexpression induced a conversion of mammary epithelial cell into mesenchymal phenotype and facilitated the branching morphogenesis of these cells in collagen gel [27]. Bcl-2 overexpression in mammary epithelial cells resulted in a complete shutdown of the expression of both E-cadherin and β1-integrin, which might facilitate the penetration of cells in the extracellular matrix. Despite the similarity in branching effects exerted by HGF and Bcl-2, their underlying mechanisms are completely different, indicating that branching morphogenesis can be achieved by multiple cellular pathways. Nevertheless, our preliminary results showed that β1-integrin expression was not altered by the transfection of Bcl-2 in MDCK cells (data not shown), ruling out the possibility that the biochemical alterations found in mammary epithelial cells can also account for renal tubulogenesis.

Besides its effects on up-regulating activity of urokinase and also on the expression of urokinase receptor mRNA, HGF also confers resistance to apoptosis in response to deprivation of cell–matrix interaction [25]. On the other hand, a recent report by Saelman and Santoro [40] demonstrated that depletion of α2 integrin by antisense α2 integrin RNA induced apoptosis in cultured MDCK cells on plates. Interestingly, when these cells were cultured in collagen gel with the addition of HGF, they developed renal cysts instead of branching tubules. These data are consistent with our observation that apoptosis is one of the essential steps for the development of renal cysts. Taken together, it is of interest to notice that both HGF and Bcl-2 confer capability for epithelial cells to survive anoikis. Therefore, prevention of apoptosis during cell–matrix interaction seems to be an essential step for renal tubule morphogenesis. Finally, during normal renal development, expression of Bcl-2 in metanephric condensates is essentially required for avoiding high levels of apoptosis and may thus create the structural base for tubule morphogenesis of the metanephron.

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APPENDIX

Abbreviations used in this article are: *bcl-2*, B-cell lymphoma-2; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HGF, hepatocyte growth factor; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline solution; uPA, urokinase-type plasminogen activator.

REFERENCES

1. Cohen JJ: Apoptosis. *Immunol Today* 14:126–130, 1993
2. Coles HS, Burne JF, Raff MC: Large scale of normal cell death in the developing rat kidney and its reduction by epidermal growth factor. *Development* 118:777–784, 1993
3. Koseki C, Herzlinger D, Al-Awqati Q: Apoptosis in metanephric development. *J Cell Biol* 119:1327–1333, 1992
4. Schumer M, Colombel MC, Sawczuk IS, Gobe G, Conner J, O'Toole K, Orson CA, Wise GJ, Buttyan R: Morphological, biochemical and molecular evidence of apoptosis during the reperfusion phase after brief periods of renal ischemia. *Am J Pathol* 149:831–838, 1992
5. Shimizu A, Yamanaka N: Apoptosis and cell desquamation in repair process of ischemic tubular necrosis. *Virchows Arch B Cell Pathol Incl Mol Pathol* 64:171–180, 1993
6. Miura M, Zhu H, Rotello R, Hartwieg EA, Yuan J: Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3. Cell* 75:651–660, 1993
7. Wang L, Miura M, Bergeron L, Zhu H, Yuan J: Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* 78:739–750, 1994
8. Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR: The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 β-converting enzyme. *Cell* 75:641–650, 1993
9. Hockenberry D, Oltvai Z, Yin X-M, Milliman C, Korsmeyer SJ: Bcl-2 functions in an antioxidant pathway apoptosis. *Cell* 75:241–251, 1993
10. Tsujimoto Y: Overexpression of the human Bcl-2 gene product results in growth enhancement of Epstein-Barr virus-immortalized B cells. *Proc Natl Acad Sci USA* 86:1958–1962, 1989
11. Vaux D, Cory S, Adams J: Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre B-cells. *Nature* 335:440–442, 1988
12. Grantham JJ: Fluid secretion, cellular proliferation and pathogenesis of renal epithelial cysts. *J Am Soc Nephrol* 3:1843–1857, 1993
13. Woo D: Apoptosis and the loss of kidney tissue in polycystic kidney diseases. *N Engl J Med* 333:18–25, 1995
14. Kamada S, Shimono A, Shinto Y, Tsujimura T, Takahashi T, Noda T, Kitamura Y, Kondoh H, Tsujimoto Y: Bcl-2 deficiency in mice leads to pleiotropic abnormalities: Accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. *Cancer Res* 55:354–359, 1995
15. Nakayama K, Nakayama K, Negishi I, Kuida K, Sawa H, Loh DY: Targeted disruption of Bcl-2αβ in mice: Occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. *Proc Natl Acad Sci USA* 91:3700–3704, 1994
16. Veis DJ, Sorenso CM, Shutter JR, Korsmeyer SJ: Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* 75:229–240, 1993
17. Mangoo-Karim R, Uchic M, Lechene C, Grantham JJ: Renal epithelial cyst formation and enlargement in vitro: Dependence on cAMP. *Proc Natl Acad Sci USA* 65:6007–6011, 1989
18. Mcateer JA, Evan AP, Gardner KD: Morphogenetic clonal growth of kidney epithelial cell line MDCK. *Anat Rec* 217:229–239, 1987
19. Montesano R, Schaller G, Orci L: Induction of epithelial tubular morphogenesis in vitro by fibroblast derived soluble factors. *Cell* 66:697–711, 1991
20. Wang AZ, Ojakian GK, Nelson WJ: Steps in the morphogenesis of a polarized epithelium I: Uncoupling the roles of cell-cell and cell substratum contact in establishing plasma membrane polarity

in multicellular epithelial (MDCK) cysts. *J Cell Sci* 95:137–151, 1990
21. Mcateer JA, Cavanagh TJ: Medium hydrated collagen gel as an explant support in organ culture. *J Tissue Cult Methods* 7:117–122, 1982
22. Tang MJ, Cheng YR, Lin HH: Role of apoptosis in growth and differentiation of renal proximal tubule cells in primary cultures. *Biochem Biophys Res Commun* 218:658–664, 1996
23. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C: A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flowcytometry. *J Immunol Methods* 139:271–279, 1991
24. Lee YC, Lin HH, Tang MJ: Glucocorticoid upregulates Na,K-ATPase α and β mRNA via an indirect mechanism in proximal tubule cell primary cultures. *Am J Physiol* 268:F862–F867, 1995
25. Frisch SM, Francis H: Disruption of epithelial cell–matrix interactions induces apoptosis. *J Cell Biol* 124:619–626, 1994
26. Lin HH, Yang TP, Jiang ST, Liu HS, Tang MJ: Inducible expression of Bcl-2 by the lac operator/repressor system in MDCK cells. *Am J Physiol* 273:F300–F307, 1997
27. Lu PJ, Lu QL, Rughetii A, Taylor-Papadimitriou J: Bcl-2 overexpression inhibits cell death and promotes the morphogenesis, but not tumorigenesis of human mammary epithelial cells. *J Cell Biol* 129:1363–1378, 1995
28. Hall HG, Farson DA, Bissell MJ: Lumen formation by epithelial cell lines in response to collagen overlay: A morphogenetic model in culture. *Proc Natl Acad Sci USA* 79:4672–4676, 1982
29. Schwimmer R, Ojakian GK: The α2β1 integrin regulates collagen-mediated MDCK epithelial membrane remodeling and tubule formation. *J Cell Sci* 108:2487–2498, 1995
30. Coucouvanis E, Martin GR: Signals for death and survival: A two-step mechanism for cavitation in the vertebrate embryos. *Cell* 83:279–287, 1995
31. Meredith J, Fazeli B, Schwartz M: The extracellular matrix as a survival factor. *Mol Biol Cell* 4:953–961, 1993
32. Tang MJ, HU JJ, Lin HH, Chiu WT, Jiang ST: Collagen gel overlay induces apoptosis of epithelial cells in cultures. *Am J Physiol Cell* 275:C921–C931, 1998
33. Re F, Zanetti A, Sironi M, Polenterutti N, Lanfrancone L, Dejana E, Colotta FM: Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. *J Cell Biol* 127:537–546, 1994
34. Ekblom P, Weller A: Ontogeny of tubulointerstitial cells. *Kidney Int* 39:394–400, 1991
35. Lebrun DP, Warnke RA, Cleary ML: Expression of bcl-2 in fetal tissue suggests a role in morphogenesis. *Am J Pathol* 142:743–753, 1993
36. Chandler DA, El-Naggar K, Brisbay S, Redline RW, McDonnell TJ: Apoptosis and expression of the bcl-2 proto-oncogene in the fetal and adult human kidney: Evidence for the contribution of Bcl-2 expression to renal carcinogenesis. *Human Pathol* 25:789–796, 1994
37. Montesano R, Muteumoto K, Nakamur TA, Orci L: Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* 67:901–908, 1991
38. Cantley LG, Garros EJG, Gandhi M, Rauchman M, Nigam SK: Regulation of mitogenesis, mitogenesis, and tubulogenesis by hepatocyte growth factor in renal collecting duct cells. *Am J Physiol* 267:F271–F280, 1994
39. Pepper MS, Muteumoto K, Nakamura T, Orci L, Montesano R: Hepatocyte growth factor increases urokinase-type plasminogen activator (u-PA) and u-PA receptor expression in MDCK cells. *J Biol Chem* 267:20493–20496, 1992
40. Saelman EUM, Santoro SA: Loss of MDCK α2β1 integrin–collagen interactions leads to impaired growth and increased apoptosis. *J Cell Sci* 108:3531–3540, 1995